PhD Thesis

Molecular biological and ecological studies on hypogeous fungi

Zsolt Merényi

Supervisor:
Zoltán Bratek PhD

PhD School in Biology, Eötvös University (Prof. Anna Erdei)
Experimental Plant Biology PhD Program (Prof. Zoltán Szigeti)

Department of Plant Physiology and Molecular Plant Biology
Eötvös University

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Introduction

The cultivation of edible ectomycorrhizal mushrooms has just began 230 years ago, with the cultivation of some hypogeous 'true truffle' (*Tuber* spp.) in orchard. These truffles became the most intensively researched ectomycorrhizal mushrooms, due to their economic and gastronomic value. Ecological impact of hypogeous fungi is also underlined by their often recognized high prevalency. Nonetheless, there are many unanswered questions regarding to the ecology, biotic and abiotic environmental demands of these species. Our knowledge is even more incomplete about the taxonomic status of the economically less valued hypogeous fungi, as well as their phylogeography not to mention their ecological and environmental demands. Studying hypogeous fungi is important for assessing their conservation status and for the development of rare species' protection strategies. Furthermore it can contribute to improve the methodology of the highly valued truffle cultivation for instance recognizing the contaminant mycorrhiza-forming fungal species.

Fruitbodies originating different areas are important for taxonomic investigations. These specimens are accessible from collections (mycotheca), where fruitbodies had been deposed, preserved and stored for later examinations. Mycotheca provide relevant information even for ecological, population genetic, phylogeographic and conservation biology studies of species. The exploration of hypogeous funga in the Carpathian-Pannonian region especially in Hungary has a special impact due to the work of Hollós (1911) and Szemere (1970) in the last century, and due to the expanding hypogea collection of the First Hungarian Truffle Society (FHTS) from the last 25 year.

In many cases the species boundaries are modified by the molecular phylogenetic tools. Conflicts between different species concepts caused an unexpectedly high rate of change. The differences between the species concepts based on morphological and molecular criteria are often caused by cryptic and pseudocryptic phenomena (two or more distinct species classified as a single species because there are no or subtle visible morphological differences between them, Bickford *et al*. 2007). Recently, a huge increase can be observed in detecting morphologically similar species, but not typically in the case of hypogeous fungi.

Aims

- Creation of a database with the data of FHTS mycotheca, including the corresponding data of pedological and coenological surveys
- Evaluation of the database and investigation of changes of hypogeous funga
- Monitoring of hypogeous fungi, especially rare or new species for a certain area
- Phylogenetic and morphological revidiation of *Tuber regianum* aff. materials
- Introduction of the phylogenetic and/or morphologic results of the genus *Genea* and Rufum group which were obtained in international cooperation.
- Detailed phylogenetic and phylogeographic examination of *T. brumale*, thought to be an invasive species
- Investigation of the delimitation of pseudocryptic species presenting example *T. brumale* aggr.

Materials and methods

A hypogea-database was created with the data of FHTS collection using Microsoft Access database engine software, which could be used for the complex queries. Continuous monitoring of the hypogeous fungi was performed by our working group with the help of trained truffle-hunting dogs. Data of collections, micro- and macromorphology, coenology and plant syntaxonomy and pedology are included in different tables of the database. Also, additional fruitbodies originating from other countries, and sequences from international nucleotide database were also used on the studies of the chosen taxa. χ^2-, and Fisher's exact test were performed for the comparison of the data frequencies between the two periods (collection data of László Hollós and László Szemere with ours) in order to evaluate the changes of hypogeous funga.

We examined the following species from the taxa with few data or new species for a certain area: *Tuber foetidum* Vittad., *Elaphomyces persoonii* Vittad., *Gymnomyces* sp. *Tuber regianum* aff. The first three taxa were only identified with morphological and/or sometimes with even molecular based tools (BLAST and Neighboor Joining, Maximum Likelihood phylogenetic tree). On the other hand *T. regianum* aff. specimens were put under detailed phylogenetic analysis (ITS LSU and PKC loci; ML and Bayesian analysis) and morphological examinations including samples from all known distributional areas (countries).

In the case of hypogea taxa requiring molecular taxonomic revision preliminary results of the genus *Genea* and Rufum group are presented. We performed sequencing the ITS region of 30 *Genea* samples originating the Carpatho-Pannon region, morphological examination on specimens belonging to new species and divergence-time estimation (BEAST) on genus level. In order to estimate the potential species richness of Rufum group, Automatic Barcode Gap Discovery (ABGD) was used on sequences which were obtained in our research group (n=104) or downloaded from GenBank (n=223).

Tuber brumale Vittad. known as a frequent contaminant species in truffle orchards was involved in multi-loci (ITS, LSU, PKC) phylogenetic (ML, Maximum Parsimony, Bayesian), phylogeographic (ITS haplotype network) analysis based on 196 samples from all over Europe to clarify its Carpathian Basin presence. The detailed morphological examination of the *T. brumale* agg. was conducted for nearly one hundred samples using 19 direct or derived characters. The delimitation of morphologically similar but phylogenetically distinct, cryptic/pseudocryptic species was tested by statistical comparison of the characters (normality, variability test, Student's T-, Brunnel-Munzel test), multivariate analysis (Linear Discriminant Analysis) and simulation for sampling (python scripts). Ecological description of the species aggregate was carried out with botanical, pedological and area reconstruction aspects as well.

Results and discussion

- Hypogea database was created using the data of the FHTS mycotheca, which contain about 4600 hypogeous fungi records, linked with 2462 pedological datasets, and 1146 coenological surveys. The database incorporate approximately 130 species belonging to 28 genera, but we do not have molecular taxonomic studies from several genera of them.
- From the examined 32 taxa, there were 10 which frequency has decreased significantly and 8 which frequency significantly increased till now, in contrast to the observed taxa in the first part of the 20th. century. This trend can mainly be explained by the collection methodological and cultural changes. Hence, there are hypogea species preferred by dogs (DPH) and non-preferred species too (eg. *Elaphomyces* spp.) (Bratek et al. 2013)
- *Tuber foetidum* was reported from the low-mapped Finland first, based on morphological and DNA analyses. At the same time it also represented the northernmost record for the species.

- From the more intensively monitored Hungary, we found *Gymnomyces* sp. firstly, what was confirmed by morphological determination.
- Six new localities and twelve new records of the protected *Elaphomyces persoonii* were found in contrast with the previously single known locality in Hungary, what resulted a locally distributed, not too rare species in Tokaj–Zemplén Mountains
- We delimited a new species for science in Hungary from the rare *Tuber* species. The new species together with *T. regianum* Montecchi & Lazzari and *T. bernardinii* Gori might form a new, the twelfth species group of the genus *Tuber*, based on comparative molecular phylogenetic and morphological examinations, what were performed for the first time at this species group. Due to the modified species boundaries, the distributional areas of the previously described two species were rewritten and it seems that *T. regianum* shows a strong host preference towards *Fagus sylvatica*
- We cooperated in providing sequences and morphological measurements for the descriptions of three new *Genea* species (*G. compressa* Z. Merényi, J. Cabero & Moreno*, G. pseudobalsleyi* Agnello, Bratek & J. Cabero, és *G. pseudoverrucosa* Bratek, Konstant. & Van Vooren) from the nine newly deliminated species (Alvarodo *et al.* 2014). Based on these results, in the Carpathian Basin there are at least seven distinct *Genea* species. We attempted to estimate the divergence time of the genus *Genea* and its major lineages based on secondary calibration approach. The first divergence event within *Genea* occurred in/during Cretaceous about 76.5 Mya (95% HPD: 42.9-112,7 Mya) at the same time the radiation of several infrageneric lineages of *Tuber* (Bonito et al 2013).
- Automatic Barcode Gap Discovery (ABGD), depending on the alignment and inter- and intraspecific threshold, classified the 327 ITS sequences from the Rufum group into 21-33 potential species. Classification with larger group number seems to be more probable, based on the examinations of different clusterings, and different methods (ML; ABGD). In the Rufum group, examining 78 specimens, it can be concluded that at least 6-8 potential species might be in Hungary. This is more than the half of the number of species in Rufum group estimated in Europe.
- Phylogenetic and phylogeographic analyses of *T. brumale* were carried out in this study using 196 samples identified as *T. brumale* or *T. brumale* var. *moschatum* attempting to cover the entire biogeographic area of this species. As a results of this, two distinct species (Clade A and B) diverged 17.9 Mya were identified but they are not identical with the former distinction of *T.*

brumale and *T. moschatum* Bonnet. Species level distinction was confirmed by the reciprocal monophyly for all loci (ITS, LSU and PKC); by the significant nucleotide difference between the ITS region of the two clades (mean 9.3% SD: 0.18%) and by their sympatric distribution as well.

- Two remarkably different ITS and LSU haplotype groups (haplogroup I. and II.) were also detected within *T. brumale* s. s. what was unsupported by the result of PKC locus. Nucleotide distance between the two groups was 2.02%, which displayed 16 mutation steps far from each other on the haplotype network. The two main phylogeographic groups show east-west separation with a zone of overlap in the Carpathian Basin, suggesting that they survived the latest glacial period in separate refugia.
- Due to the geographically widespread sampling and the big sample size, we present a higher ITS variability in *T. brumale* s. l. than was previously believed. Our data show that intraspecific ITS variabilities of clades A and B are 0.91 and 0.07 %, respectively, meaning that clade B does harbour low variability while that of clade A is relatively high. Intraspecific ITS variabilities of haplogroups I and II are 0.032 and 0.201 %, pointing out that intergroup differences contribute the most to the genetic variation found in clade A. The haplotype diversities are the follows: A/I Hd=0.29; A/II Hd=0.72; B Hd=0.53.
- Observed geographical patterns, ITS haplotype network topology, and the results of the neutrality tests indicate that the haplogroup I. survived the last glacial in Western European refugia, where it might have suffered a bottleneck. Haplogroup II is more diverse, its nucleotide and haplotype variability is higher and the structure of its haplotype network is more complex. This greater diversity and current distribution could be a result of surviving the last glacial in South-Eastern European refugia. The low nucleotide and haplotype diversity of clade B can indicate a strong bottleneck effect, however, the results of neutrality tests do not support this assumption. A more limited distribution, than the predicted, caused by historical events in the quaterner can suggest that clade B is distributed solely in the Carpathian Basin indeed and represents a fungus endemic to this region.
- Preliminary morphological measurements on *T. brumale* aggr. did not show any phenotipical differences between the two phylogenetic species, which would have indicated them as cryptic species. We managed to find overlapping characters which show significant difference between the two species by detailed examinations with increasing the number of the characters and the sample size. Linear Discriminant Analysis estimated a discriminant function and character combination, including exoperidial cell size, warts diameter, and R3 (ratio of 3-spored asci).

This derived character resulted clear separation on the 95.2% of the samples. Hence the two members of *T. brumale* aggr. can rather be considered as pseudocryptic than cryptic species.

- Trying different combinations of pedological variables the most effective separation was achieved by the combination of Mn, Mg and Cu values. According to this result, with the other dataset (n=72) the classification success was 76.4% based on only pedological variables (Mn, Mg, Cu contents) and it was 94% based on only morphological variables (average size of peridial cells, average diameter of warts, and R3). The most effective separation (97.2%) was achieved by the combination of the best morphological variables expanded with pedological variables (Mn, Mg and Cu contents). It highlights the efficiency of the integrative taxonomy, which combine different molecular, morphological and ecological data.
- It can be concluded from the simulation of the pseudocryptic species delimitation (like *T. brumale* clade A and clade B) that a much larger number of samples is necessary for the discovery of the real differences between closely related species. There would have a non-negligible chance that this phylogenetically supported species were separated with totally wrong characters, under a small sample size.

Conclusions

Evaluation of the collection data was facilitated by creating a database from the data of FHTS mycotheca, covering the last 25 years. Due to the lack of the molecular phylogenetic studies on genus level, there are still a lot of ambiguous taxa in the database. The distribution of the collection samples is inhomogeneous because it is mainly influenced by the research intensity. So often the locations of the most intensively investigated areas, where the economically important species are harvested. While the changes of the occurrence frequency can be most obviously explained by the changes in the collecting methods. Fewer data can be available for those species, which were not investigated in the whole (*Tuber foetidum*) or partial (*Elaphomyces persoonii*) area, which are a newly appeared species on the area (*Gymnomyces* sp.), or which are extremely rare (Regianum group). It is clear from the above mentioned examples, that only continuous collecting work over a long period could provide sufficiently accurate information about the species richness of an area or the real species occurrence frequency thereby define the protection status of these

fungi. Molecular phylogenetic analyses of the genus *Genea*, Regianum and Rufum groups, and *T. brumale* aggr. confirm that resolve the taxonomy of species can only be performed reliably when the geographical sampling is broad enough. The previously not assumed high levels of hidden diversity emphasizes the contradictions between morphological and phylogenetic species concepts, which leads to more and more phylogenetic species discovered by molecular tools, but there are proportionally fewer described species, among them (Hutsemékers et al. 2012, Oliver & Lee 2010). Investigating the morphological characters of *T. brumale* aggr. species, it can be concluded that phenotypic plasticity has a non-negligible influence for the pseudocryptic, closely related species characters, which are often continuous and overlapped. It seems to be that the identification keys with discrete characters can often resolve the entity only on the level of species complex, and the "real" species delimitations are only possible by using combinations of the continuous, overlapped characters, with the approach of integrative taxonomy. Moreover a much larger number of samples is necessary for the discovery of the real differences between closely related species. In conclusion, the long term, intensive monitoring, complemented with molecular phylogenetic identification might contribute to better estimation of species richness in an area or in a taxon.

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